

A Study of the Role of Nitric Oxide in the Mechanism of Action of Hydroalcoholic Extract of Saffron (*Crocus sativus*) on the Electrophysiological Properties of the Rabbit Atrioventricular Node

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Abstract

Biologically active substances of plant origin represent an essential branch of modern cardiovascular pharmacotherapy. Furthermore, drugs of plant origin have the advantage of weaker adverse effects and lower prices than synthetic drugs. Pharmacological studies and traditional medical literature point to the anti-ischemic and hypotensive effects of the *Crocus sativus* L. (Iridaceae). The major goals of the present study were: (1) to determine the negative dromotropic properties of a hydroalcoholic extract of saffron on an isolated AV node and (2) to establish the role of nitric oxide in the mediating effects of saffron on the electrophysiological properties of the AV node. This was an experimental study. Selective stimulation protocols were used to independently quantify AV nodal recovery, facilitation, and fatigue. We used isolated perfused rabbit AV node preparation, in three groups (N=32); in each group, we assessed the plant's effect in comparison with the control. In the pilot study, we used different concentrations (A=9×10⁻² mg/l, B=19×10⁻² mg/l, and C=27×10⁻² mg/l) to select the optimum concentration (19×10⁻² mg/l) of the hydroalcoholic extract of Saffron. Saffron has a depressant effect on basic and rate-dependent properties of the AV node. We observed an increasing AVCT (38.8 ± 4 to 41.7 ± 4 msec) and FRP (157.6 ± 3 to 163.7 ± 4 msec). Also saffron increased the amount of facilitation and the magnitude of fatigue (5.9 ± 0.3 to 11.1 ± 1 msec). The NOS inhibitor (L-NAME) has a preventative effect on the depressant effect of saffron on AVCT and FRP.

INTRODUCTION

The atrioventricular node (AV node) is the most significant determinant of the normal variability of the PR interval, which is essential for optimal ventricular pumping, and serves as a safeguard to slow or block premature atrial impulses that could trigger ventricular fibrillation (Billette et al., 1995). The wide varieties of delay that the AV node can generate in response to an increased rate are explained by dynamic interactions among the three intrinsic properties of recovery, facilitation, and fatigue (Billette et al., 1994). A large and increasing number of patients in the world use medicinal plants and herbs for health purposes. Therefore, scientific scrutiny of their therapeutic potential, biological properties, and safety would be useful in so users may make wise decisions about their use (Rios et al., 1996). From ancient times to the present, *Crocus sativus* L. (Iridaceae), a plant well known as saffron has been

used as a spice for flavoring and coloring food. In folklore medicine, as well as in modern drugs, saffron has been reputed to be useful in the treatment of numerous human diseases (Rios et al., 1996). Commercial saffron comprises the dried red stigma with a small portion of the yellowish style attached. This plant is cultivated in India, Kashmir, and parts of Iran (Rios et al., 1996). Saffron contains three main pharmacologically active metabolites: crocin, picrocrocin, and safranal (Rios et al., 1996). Previous studies have shown the anti-hypertensive and anti-ischemic properties of saffron (Fatehi et al., 2003). Alcoholic and aqueous extracts of saffron were able to reduce blood pressure in rats; in addition, an aqueous extract of saffron could inhibit smooth muscle contraction, induced by 0.1 μ mol of epinephrine (Fatehi et al., 2003). Until now, there have not been any studies of the effects of saffron and specially the role of nitric oxide in the saffron's effects on the electrophysiological properties of the isolated, perfused rabbit atrioventricular node. Crocetin from saffron's extract inhibits mRNA expression of inducible nitric oxide synthase in the liver of rats with hemorrhagic shock (Yang et al., 2006). The aims of the present study were: (1) to assess the electrophysiological effects of a hydroalcoholic extract of saffron on the isolated perfused rabbit and (2) to establish role of nitric oxide on the mediating nodal effects of saffron.

MATERIALS AND METHODS

Saffron was purchased from a commercial brand (Novin Zaferan Co.) in Mashhad, Iran. A voucher specimen (150-0319-02) was designated for the saffron used in this investigation by the Mashhad University of Medical Sciences in the Khorasan province in eastern Iran. The stigmas were collected in the autumn of 2004 and air-dried at 40 °C. The saffron powder was dissolved in 20ml of ethanol (80 %). The solution was kept in the dark for four days. The prepared solution was filtered and was concentrated at a temperature of 30 °C to 40 °C using a vacuumed rotatory. The final concentration of the saffron extract was 68 %. Pure safranal (FULKA Co.) was used as a standard to determine the safranal concentration in the saffron extract.

High performance liquid chromatography (HPLC) analysis of the saffron extract using a UV detector with a wavelength of 308 nm showed the presence of safranal with a concentration of 5.09 %. The HPLC fingerprint of the ethanol extract is shown in Fig. 1. [HPLC ODS; Solvent: water + acetonitrile (76 % v/v); Flow rate 0.8 ml/min; Injection 20 μ l ethanol extract].

All experiments were performed in vitro on isolated perfused rabbit cardiac preparations obtained from hearts on Newsland white male rabbits of weighing 1.0 to 1.3 kg. Anesthesia was induced with a pentobarbital (35 mg/kg) injection in the ear vein, and heparin (200 IU/kg) was used as anticoagulant. A lateral thoracotomy was performed, and the heart was excised. Ethical approval and animal care were in accordance to the principles in the regulations in use at the Golestan University. Experiments were performed on isolated, perfused rabbit AV nodal preparations. The preparation, perfusion system, stimulation technique, and recording system were similar to those that have been previously described in detail (Nayebpour et al. 2001). The final preparation, which included the right atrium, area, and upper part of the interventricular septum, was mounted in a tissue bath superfused at 200 ml/min with a 6-liter volume of oxygenated (95 % O₂ -5 % CO₂) Tyrode's solution, maintained at 37 °C (pH=7.38).

The aorta was retrogradely perfused by a peristaltic pump, at a constant pressure equivalent to 60 mmHg to 80 mmHg. The composition of the Tyrode's

solution was (mmol/l): NaCl, 128.2; KCl, 4.7; CaCl₂, 2; MgCl₂, 1; 1; NaHCO₃, 25; NaH₂PO₄, 0.7; and dextrose, 11.1.

A bipolar iridium-platinum stimulating electrode was positioned on the upper atrium near the sinus node, and unipolar electrograms were recorded from the low upper atrium, and His bundle. Stimulation protocols were executed by custom-made software running on a Pentium computer interfaced with a D/A converter and a stimulus isolator. Electrogram signals were filtered (30 Hz to 3 KHz) and amplified by an amplifier. Afterward, the A/D conversion data were saved on a hard disk and analyzed offline.

Stimulation Protocols Used to Quantify Recovery, Facilitation, and Fatigue

Specific stimulation protocols were used to quantify properties of AV nodal recovery, facilitation, and fatigue as previously described (Nayebpour et al., 2001). To construct the basic recovery curve, a single premature or delayed stimulus (S2) was introduced after every 10 basic stimuli (S1). The relation between the conduction time of the test beat (A2H2) and the preceding recovery time (H1A2) was established and fitted to an exponential function as previously described (Nayebpour et al., 2001). To study facilitation, the recovery curve was constructed following a facilitation-inducing short cycle introduced after the last basic stimulus. To analyze AV nodal fatigue, two series of tachycardia with a constant AA interval were initiated, and changes in the AH interval over 5 minutes at a given AA interval were observed. A recovery period of at least 5 minutes was allowed after each tachycardia for the dissipation of fatigue before the next tachycardia was initiated. The functional and effective refractory periods and Wenckebach period of the AVFRP, AVERP, and WBCL, respectively, were measured with the extra-stimulus technique, as previously described (Nayebpour et al., 2001). All the stimulation protocols were carried out under control conditions (no intervention) and in the presence of either of two groups of experiments (A-B) as follows:

Experiments A. In the first preliminary series of experiments, the effects of various concentrations of a hydroalcoholic extract of saffron ($A=9 \times 10^{-2}$ mg/l, $B=19 \times 10^{-2}$ mg/l, and $C=27 \times 10^{-2}$ mg/l) were studied separately and cumulatively on the electrophysiological properties of the AV node. These sets of experiments were used to determine the optimum concentration of saffron (19×10^{-2} mg/l) to produce the desired response.

Experiments B. In a separate series of experiments, we tested the involvement of nitric oxide (NO) in the AV nodal effects of the saffron extract by using a specific NOS inhibitor (L-NAME). Verapamil was added as a positive control to quantify the relative potency of saffron. The stimulation protocols were carried out under control conditions (no intervention) and the addition of L-NAME (75 μ mol) and the extract. Verapamil (0.1 μ m) was tested in a separate group of experiments in the absence of the *saffron* extract. The protocols were performed after perfusion periods of 30 min and 20 min for L-NAME and verapamil, respectively. The pilot study showed that 45 min of perfusion is enough to obtain steady state effects of saffron. The results reported as the mean \pm SE and comparisons among multiple groups were made by two-way analysis of variance (ANOVA) with Scheffe contrasts. Comparisons between two groups of experiments were made only with the Wilcoxon signed ranks test.

RESULTS AND DISCUSSION

The hydroalcoholic extract of saffron in a concentration of 19×10^{-2} mg/l caused a basic and rate-independent depression of the nodal parameters (Table 1; Fig. 2). The time-dependent effect of saffron appeared after 45 minutes, and reached its peak gradually in about 90 minutes (Fig. 2). This effect appeared as an upward shift of the conduction curve on both the smooth and steep portions of the curve. The minimum conduction time (AH min) was significantly increased from 32.8 ± 3.2 to 34.3 ± 3.5 msec ($p < 0.05$), while we observed a non-significant increase in AH max from 75.6 ± 8.8 to 78 ± 7.3 msec ($p > 0.05$).

The non-linear fitting of the recovery curve (Fig. 5) by using mono-exponential method showed a non-significant prolongation of the time constant of recovery from 29.8 ± 5.6 to 31.1 ± 4.6 msec ($p > 0.05$). The results of adding L-NAME (75 μ mol) showed a decrease in the depressant effect of saffron, especially on AVCT and WBCL (Table 1). The depressant effect of saffron (19×10^{-2} mg/l) on the atrionodal conduction time was 11.6 % times that of verapamil (Table.1). This means that the depressant effect of the saffron extract compared to a 0.1 μ mol concentration of verapamil is negligible (Table 1).

Saffron increased the amount of facilitation and magnitude of fatigue (Figure 4). The amount of facilitation and degree of fatigue was 6.2 ± 0.9 and 5.9 ± 0.3 msec in the control groups, and these indexes were increased by *saffron* to 9.8 ± 1.1 and 11.1 ± 1 msec, respectively. Saffron (19×10^{-2} mg/l) caused a significant increase in WBCL from 141.4 ± 4 to 148.7 ± 5 msec and FRP from 157.6 ± 3 to 163.7 ± 4 msec, respectively. Saffron showed a non-significant decrease in ERP (Figure 3). Adding L-NAME (75 μ mol) caused time-dependent depressant effects that reached a maximum in 40 minutes. In the presence of L-NAME, the inhibitory effects of saffron on the facilitation and fatigue indexes was abolished, such that the magnitude of fatigue was 12.5 ± 1.9 and 12.8 ± 2 msec, in the control and L-NAME group, respectively.

This study showed the depressant effect of a hydroalcoholic extract of saffron on the isolated rabbit AV node. These effects indicate the differential effects of saffron on the slow and fast pathways in a rate-independent manner (meaning that, the effect of saffron doesn't increase with an increasing rate of atrial stimulation). Recent studies indicate that the conduction curve of the atrioventricular node consists of two different parts. The smooth part of the curve in a long atrial stimulation is an indication of conduction through a fast pathway, and the steep portion of the curve in a fast atrial stimulation represents conduction through a slow pathway (Reid et al., 2003). In Figure 2, saffron depresses the fast pathway more than the slow pathway. The significant increase in the minimum nodal conduction time (AH min) indicates the plant's effect on the fast pathway (the transitional cells of the posterior section of the compact node). Saffron had a non-significant prolongation of maximum conduction time (AH max); however, at the same time decreasing ERP by saffron indicates its minor role on posterior nodal extension. The inhibitory effect of a hydroalcoholic extract of saffron in having a non-significant increase in the time constant of recovery explains the rate-independent effect of the saffron extract.

The atrioventricular node acts as a control center for the ventricular impulses during tachyarrhythmia. In a functional model, the reason for the delay in nodal conduction was due to the specific nodal intrinsic properties (Billette et al., 1994). Therefore, by understanding the mechanism of above phenomenon, we can explore the anti-arrhythmic effect of endogenous and exogenous substances. The effect of saffron on fatigue represents the potential role of this plant in preventing arrhythmia. In the fatigue protocol, the tissue sample was stimulated by various rates similar to supraventricular tachyarrhythmia. The increase in the fatigue index by saffron reflects

the plant's role in decreasing the excitability of the distal node and an increase in the protective role of the atrioventricular node.

Previous studies have demonstrated the role of transitional cells proximal to the node in the facilitation mechanism (Mazgalev et al. 1997). We can assume that saffron probably exerts its effect on the proximal node. The fatigue mechanism is related to the prolonged FRP of compact nodal cells (Billette et al. 1995); therefore, the prolongation of fatigue by saffron is due to effect of this plant on the compact nodal cells of the distal AV node. In the heart, NOS enzymes (NOS₁, NOS₃) have been identified in cardiac conductive tissue, and the atrioventricular node (Han et al. 1996). There is strong evidence about role of nitric oxide in the physiological and pathological regulation of the AV node (Balligand et al. 1997). The results of the present study confirm that nitric oxide mediates at least in part the inhibitory effect of saffron on the AV node. A similar role of nitric oxide has been demonstrated for the attenuation caused by acetylcholin and adenosine of β -Ical in rabbit-isolated AV nodal myocytes (Martynyuk et al. 1997; Han et al., 1996). Indeed, the anti-arrhythmic effect of adenosine is related to the release of nitric oxide and consequently, the inhibition of β -Ical. (Martynyuk et al., 1997). Aside from L-NAME's inhibitory effect on the depressive effect of saffron, the NOS-inhibitor (L-NAME) itself slightly prolonged the AH interval and significantly increased WBCL and FRP. A similar prolongation of conduction time in response to the NOS-inhibitor was observed in dogs, rats, and isolated rabbit AV nodal cells (Zima et al., 2000; Elvan et al., 1997; Muller et al., 2000). Taken together, the discrepancies suggest that additional NO-depressant mechanisms or an intrinsic inhibitory activity of L-NAME are possible mechanisms of L-NAME in the regulation of AV nodal function.

This research has explained the role of saffron to enhance the protective role of the atrioventricular node against supraventricular arrhythmia. The results from this research showed the non-specific effect of saffron on the transitional cells of the fast nodal pathway, which was manifested as a rate-independent increase of basic and functional (facilitation and fatigue) parameters of the atrioventricular node. Further investigations are required to reveal the cellular mechanism of saffron on the AV node and the potential use in treating supraventricular tachyarrhythmia in humans.

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Literature Cited

- Balligand, J.L. and Cannon, P.J. 1997. Nitric oxide synthesis and cardiac muscle, autocrine and paracrine influences. *Arterioscler Thromb Vasc Biol* 17(10):1846-1858.
- Billette, J. and Nattle, S. 1994. Dynamic behavior of the atrioventricular node: a functional model of in interaction between recovery, facilitation, and fatigue. *J. cardiovascular. Electrophysiol.* 5(1):90-102.
- Billette, J. and Shrier, A. 1995. Atrioventricular nodal activation and functional properties. In Zipes D.P., Jalife J., eds. *Cardiac Electrophysiology. From Cell to Bedside*. WB Saunders, Philadelphia. 1995: pp. 216-228.
- Elvan, A., Rubart, M. and Zipes, D.P. 1997. No modulates autonomic effects on sinus discharge rate and AV nodal conduction in open-chest dogs. *Am. J. Physiol.* 272:H263-H271.

- Fatehi, M., Rashidabady, T., Fatehi-Hassanabad, Z. 2003. Effects of *Crocus sativus* petals' extract on rat blood pressure and on responses induced by electrical field stimulation in the rat isolated vas deferens and guinea-pig ileum. J. Ethnopharmacol. 84:199-203.
- Han, X., Kobzik, L., Balligand, J.L., Kelly, R.A., Smith, T.W. and Michel, T. 1996. Nitric Oxide synthase (NOS₃) mediated cholinergic modulation of Ca current in adult rabbit atrioventricular nodal cells. Circ. Res. 78:998-1008.
- Martynyuk, A.E., Kane, K.A., Cobbe, S.M. and Rankin, A.C. 1997. Role of nitric oxide, cyclic GMP and superoxide in inhibition by adenosine of calcium current in rabbit atrioventricular nodal cells. Cardiovascular Res. 34(2):360-367.
- Mazgalev, T., Mowrey, K. and Efimov, I. 1997. Mechanism of atrioventricular nodal facilitation in rabbit heart: role of proximal. Am. J. Physiol. 272(HCP42):H1658-H1668.
- Muller S.G., Kottenberg, K., Zimmer, H.G., Noack, E. and Kojda, G. 2000. Inhibition of Nitric Oxide synthase augments the positive inotropic effect of Nitric Oxide donors in the rat heart. J. Physiol. 522(2):311-320.
- Nayebpour, M., Naseri, M. and Khorri, V. 2001. Effects of Ajmaline on rate dependent atrioventricular node properties potential role in experimental atrioventricular re-entrant tachycardia. Eur. J. Pharmacol. 412(1):77-89.
- Reid, M.C., Billette, J., Khalife, K. and Tadros, R. 2003. Role of compact node and posterior extension in direction-dependent changes in atrioventricular nodal function in rabbit. J. Cardiovasc Electrophysiol. 14(12):1342-1350.
- Rios, J.L., Recio, M.C., Giner, R.M. and Mañez, S. 1996. An update review of saffron and its active compounds. Phytother. Res. 10(3):189-193.
- Yang R, Tan X, Thomas AM, Shen J, Qureshi N. and Morrison, D.C. 2006. Crocetin inhibits mRNA expression for tumor necrosis factor- α , interleukin-1 β , and inducible nitric oxide synthase in hemorrhagic shock. J. Parenter. Enteral. Nutr. 30(4):297-301.
- Zima, A., Martynyuk, A.E., Seubert, C.N., Morey, T.E., Sumners, C., Cucchiara, R.F. and Dennis, D.M., 2000. Antagonism of the positive dromotropic effect of isoproterenol by adenosine: role of nitric oxide, cGMP-dependent cAMP-phosphodiesterase and protein kinase G. J. Mol. Cell Cardiol. 32(9):1609-1619.

Table 1. The effect of the hydroalcoholic extract of saffron (19×10^{-2} mg/l) on the basic properties of the AV node (N=32), mean \pm SEM, * $p < 0.05$, ** $p < 0.01$ compared to the control. WBCL: Wenckebach cycle length, FRP: Functional refractory period, ERP: Effective Refractory Period, Δ Verapamil: Difference between verapamil and saffron effects (percent).

	AH(msec)		WBCL(msec)		ERP (msec)		FRP(msec)	
	Control	case	Control	case	Control	case	Control	case
Saffron (19×10^{-2} mg/l)	38.8 \pm 4	41.7 \pm 4	141.4 \pm 4	148.7 \pm 5	122.6 \pm 6	121.3 \pm 6	157.6 \pm 3	163.7 \pm 4
Saffron (19×10^{-2} mg/l) + L-Name(75 μ mol)	46 \pm 8	46.3 \pm 9	155.3 \pm 3	156 \pm 3	116.3 \pm 16	114.6 \pm 16	163.3 \pm 4	168.3 \pm 1
Verapamil 0.1 μ molar	42	67	143	225	108	139	160	213
Δ Verapamil 0.1 μ molar	11.6%		0%		11.5%		6.4%	

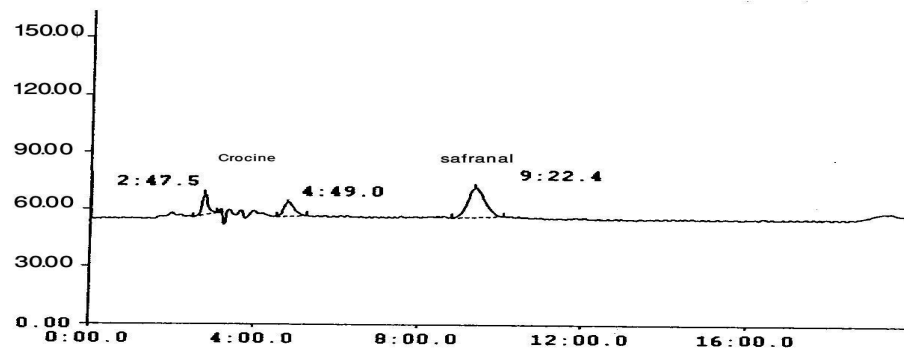


Fig. 1: HPLC profiles of ethanolic extract of *Crocus sativus* at 318 nm. The major peaks in the extract were assignable to safranal.

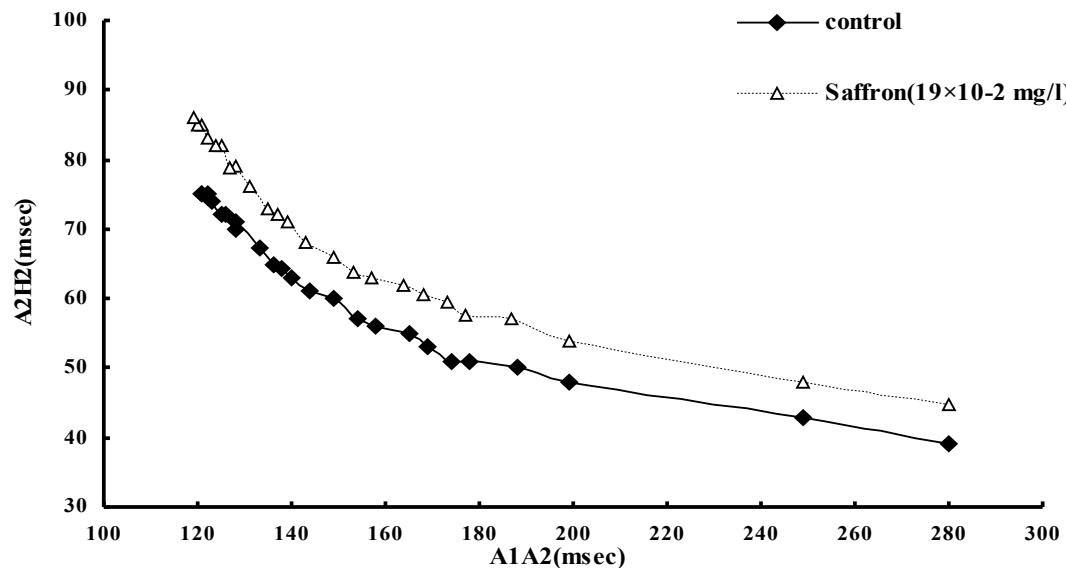


Fig. 2. The conduction curve that was obtained during the recovery stimulation protocol in one reparation in the presence of the alcoholic extract of saffron (19×10^{-2} mg/l). There is an upward shift of recovery curve.

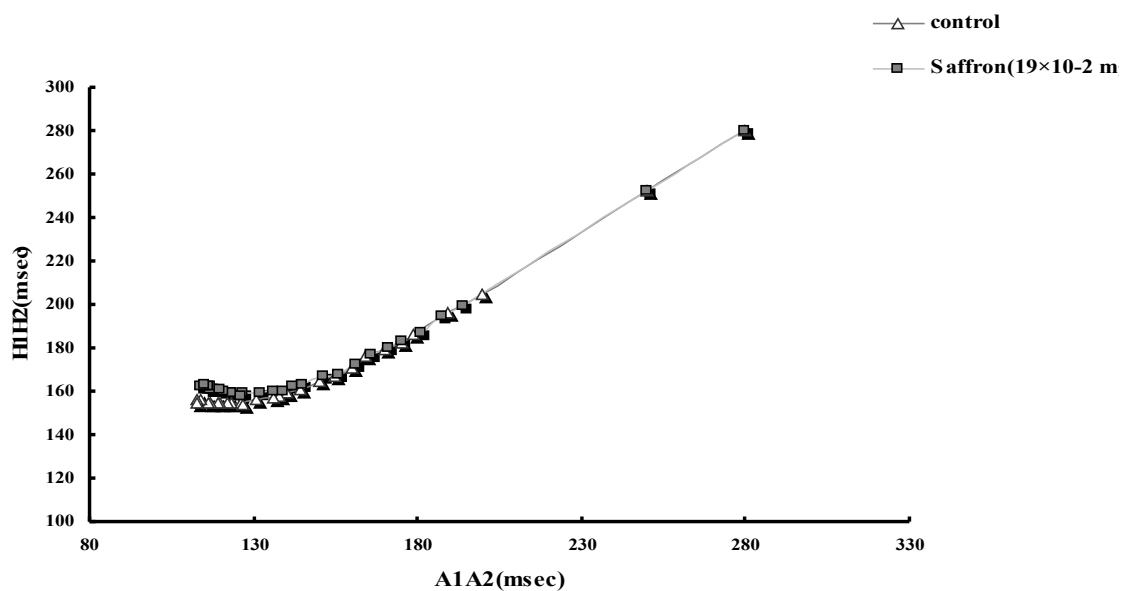


Fig. 3. The refractory curve that obtained during the recovery protocol in one preparation in the presence of the alcoholic extract of saffron (19×10^{-2} mg/l). There is an upward shift in the refractory curve that indicated distal depression of AV node.

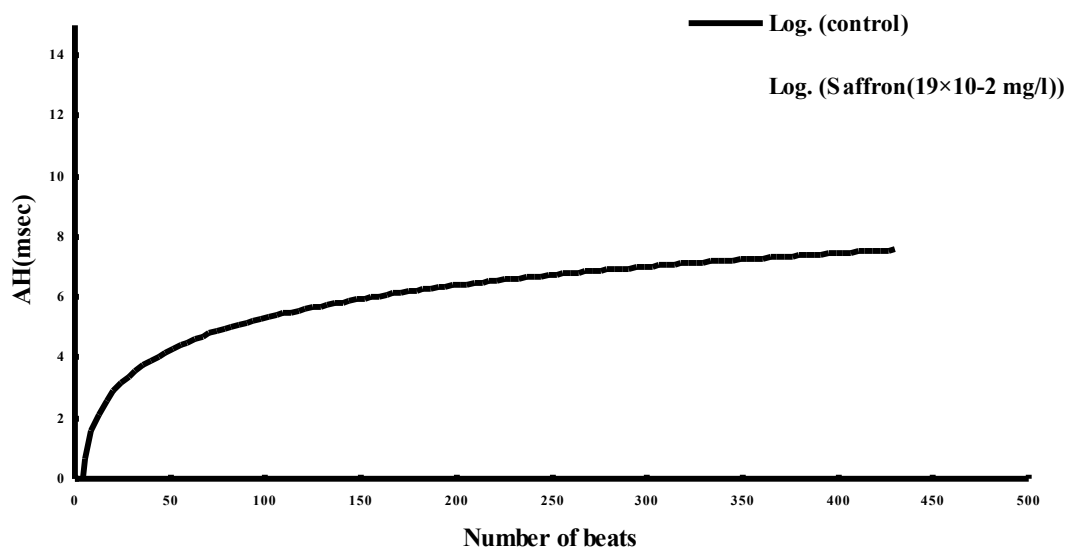


Fig. 4. The fatigue curve that was obtained during the fatigue protocol with a tachycardia cycle length of 200 msec in one preparation. Saffron caused an upward shift of the control curve.log: Mathematical logarithmic fitting of fatigue curve

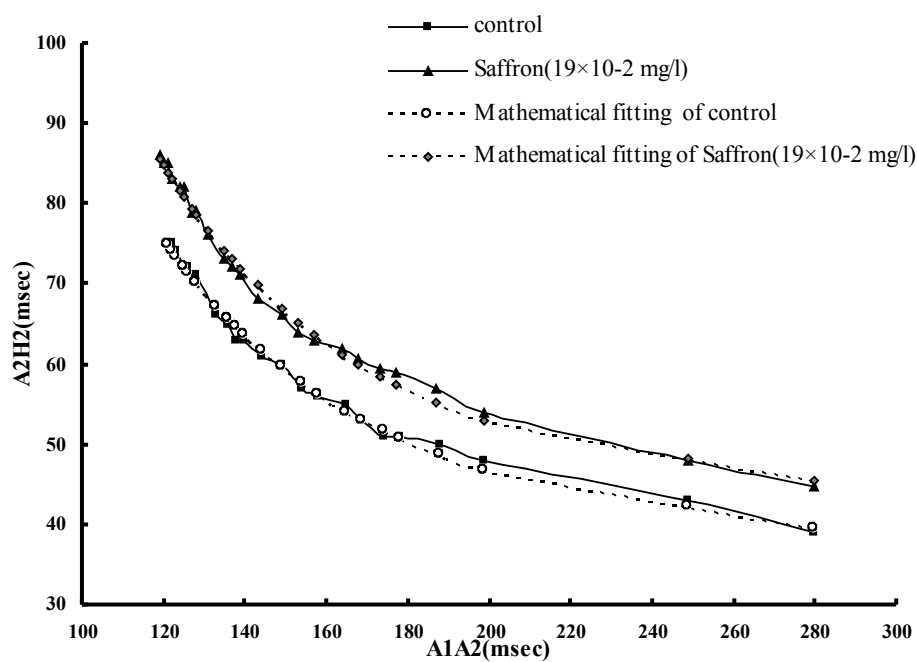


Fig. 5. Plot of a non-linear curve fitting of the recovery curve using the Marquart method in one preparation in the presence of saffron